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The role of oxidised regenerated cellulose/collagen in chronic wound repair and its potential mechanism of action

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Abstract

Normal wound healing is a carefully controlled balance of destructive processes necessary to remove damaged tissue and repair processes which lead to new tissue formation. Proteases and growth factors play a pivotal role in regulating this balance, and if disrupted in favour of degradation then delayed healing ensues; a trait of chronic wounds. Whilst there are many types of chronic wounds, biochemically they are thought to be similar in that they are characterised by a prolonged inflammatory phase, which results in elevated levels of proteases and diminished growth factor activity. This increase in proteolytic activity and subsequent degradation of growth factors is thought to contribute to the net tissue loss associated with these chronic wounds.

In this study, we describe a new wound treatment, comprising oxidised regenerated cellulose and collagen (ORC/collagen), which can redress this imbalance and modify the chronic wound environment. We demonstrate that ORC/collagen can inactivate potentially harmful factors such as proteases, oxygen free radicals and excess metal ions present in chronic wound fluid, whilst simultaneously protecting positive factors such as growth factors and delivering them back to the wound.

These characteristics suggest a beneficial role for this material in helping to re-balance the chronic wound environment and therefore promote healing.

Keywords: Protease; Free radicals; Iron; Growth factors; Wound healing

1. Introduction

Chronic wounds such as pressure sores, diabetic foot ulcers, venous leg ulcers and other delayed wounds can remain unhealed for weeks, months or even years. This represents a huge financial burden to the health care system. Contributing to these costs are treatment regimes that are expensive and/or ineffective. In general, these therapies are aimed at eliminating tissue trauma, reducing tissue ischaemia and minimising infection while providing a moist wound healing environment considered beneficial for optimal healing [1]. More recently, growth factor therapies, such as REGRANEX have been developed, however, the addition of growth factors to chronic wounds has met with limited success. This is thought to be due to a prolonged inflammatory state generating a hostile wound environment by stimulating an overproduction of proteases and free radicals.

Many studies have investigated the role of proteases in human chronic wounds, and have concluded that elevated proteolytic activity contributes to their chronicity [2,3]. While controlled degradation is necessary for normal wound repair, excess proteolysis is

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considered detrimental, and may result in the degradation of the extracellular matrix [4,5] and key functional molecules such as growth factors [6–8], their cell surface receptors and integrins [9,10]. Growth factors such as PDGF, TGBβ and bFGF are pivotal in normal wound repair, driving cell migration, proliferation, protein synthesis, matrix formation, and generally controlling the repair process [6,7]. However, since these growth factors are proteinaceous molecules they are susceptible to proteolytic degradation, an effect observed in chronic wounds [7,8,11,12]. In addition, the biological activity of many of these growth factors is enhanced by the presence of specific matrix proteins [13], thus, the degradation of either the growth factors or the extracellular matrix by excess proteases in the wound could lead to diminished growth factor activity and delayed healing.

Other potentially damaging components of the chronic wound environment include extracellular metal ions and free radicals. Elevated concentrations of active oxygen species such as hydroxyl radicals ($^{\bullet}OH$), hydroperoxyl radicals ($^{\bullet}OOH$), superoxide anions ($^{\bullet}O^{-}$) and other free radical species such as nitric oxide (NO) have been implicated in the pathology of a number of diseases including, arthritis, lipodermatosclerosis, periodontal disease and other inflammatory conditions [14]. The presence of oxygen free radicals is necessary in the early stages of wound healing to initiate the inflammatory response, attract macrophages to the wound site and promote the respiratory burst. At low concentrations (10^{-8} to 10^{-6} molar) oxidizing species with paired electrons, such as hydrogen peroxide (H_2O_2), have been shown to have a positive effect in acute wound repair, stimulating fibroblast proliferation [15]. However, their continued presence is thought to be detrimental to the healing process [14]. Studies have shown that reactive oxygen species (ROS) can induce matrix metalloproteinases production and in severe cases can result in tissue necrosis and permanent tissue damage [16]. In chronic wounds, the presence of H_2O_2 can induce the production of oxygen free radicals, an effect facilitated by the presence of excess iron. Usually iron is stored in the ferric form (Fe^{3+}) bound to protein chelators such as haemosiderin and ferritin. However, if the protein binding capacity is exceeded, or if reducing agents are present, ferric iron (Fe^{3+}) is reduced to the ferrous form (Fe^{2+}), which acts as a catalyst for the Fenton reaction converting H_2O_2 to ROS. These ROS subsequently cause protein and lipid peroxidation and DNA damage, leading to cell disruption, tissue damage and cell death [17]. The detrimental effect of excess iron, and subsequent free radical production, has been demonstrated both in vitro [16,18,19] and in vivo [20,21]. Clinical studies have shown that iron in the form of haemosiderin is deposited in legs of patients with venous hypertension and it has been hypothesised that this may be a causative factor in ulcer formation [22]. Furthermore, it has been demonstrated that desferroxamine, an iron chelator, improved skin flap survival in a porcine model [23], and that free radical scavengers, SOD or catalase, can reduce thermal injury in rat skin [24]. This has led to the use of free radical scavengers for treatment of human chronic wounds and has resulted in some success. Topical allopurinol or dimethyl sulfoxide given to 88 ulcer patients enrolled in a 3-month double-blinded study showed a significant increase in healing rate [25]. Oral pentoxyfylline, was used to treat 80 venous ulcer patients in a 6-month double-blinded study; 23 of 38 healed in the treated group, in comparison to the placebo group where only 12 of 42 patients healed [26].

This study describes the evaluation of a new proprietary wound treatment, comprising oxidised regenerated cellulose and collagen (ORC/collagen). This biomaterial was designed to modify the chronic wound environment by significantly reducing harmful factors such as proteases, oxygen free radicals and excess metal ions, whilst simultaneously protecting positive factors such as growth factors and delivering them back to the wound. This work suggests that this new wound therapy can address the imbalance between tissue synthesis and degradation in chronic wounds, and thereby provide a more efficacious treatment for chronic wounds.

2. Materials and methods

2.1. Preparation of ORC/collagen sponges

Purified collagen was prepared by extensively washing the lower split (corium) of bovine hide. The dermis was cut into 10 cm squares and washed in a 0.4% (v/v) sodium hydroxide solution for 10 days, to remove residual hair and non-collagenous

components. After washing, these squares were chopped and homogenised in 0.05 M acetic acid to give a final concentration of 4% (w/v) solids. ORC powder, produced by milling SURGICEL* cloth (Ethicon Inc., New Jersey, USA), was added to the collagen suspension at a weight ratio of 45:55 ORC: collagen to give a total solids concentration of 1.0% (w/v), and the mixture homogenised. The suspension was degassed in a vacuum oven for 10 min, and then poured into a tray and frozen to −40 °C. The frozen suspension was then freeze-dried and dehydrothermally cross-linked using a programmable freeze-drier with a temperature ramping facility. The freeze-dried product was packaged, sterilised by γ-irradiation and stored at room temperature until testing.

2.2. Chronic wound fluid collection and elution

Wound fluid was collected from patients with diabetic foot ulcers of greater than 30 days duration and a surface area larger than 1 cm^2. Patients were excluded if the target wound showed any signs of infection or if exposed bone with positive osteomyelitis was observed. Informed consent was obtained from patients or authorised representative prior to study enrolment and the institutional review board and ethic committee at each participating study centre approved the protocol. The study was conducted in accordance with both the Declaration of Helsinki and Good Clinical Practice.

Wound fluid was collected by absorption onto a piece of RELEASE* dressing (Johnson & Johnson Medical Ltd., Ascot, UK), which was placed, directly on each diabetic foot ulcer. Briefly, the RELEASE* dressing was cut to the size of the wound, placed in contact with the ulcer bed for 24 h, and covered with BIOCLUSIVE* an occlusive film (Johnson & Johnson Medical Ltd., Ascot, UK). The dressing was then removed and frozen at −70 °C until elution of wound fluid.

Wound fluid was eluted from the RELEASE* dressing by incubating the sample in 1 ml of wash buffer/cm^2 dressing (Wash buffer: 0.1 M Tris/HCl, pH 7.4 containing 0.1% Triton X-100). To allow maximum recovery of fluid the sample was incubated for 2 h at 4 °C after which the dressing was compressed against the side of the container and the eluent carefully removed, aliquoted and frozen at −70 °C until testing for protease activity. Once an aliquot was thawed it was used immediately and any remaining sample discarded.

2.3. Inactivation of collagenase-like activity in wound fluid

The ability of ORC/collagen to reduce protease activity was assessed by incubating the test material in an extract of human chronic wound fluid and measuring residual proteolytic activity. To evaluate if this property was specific to ORC/collagen a comparison was made with gauze (SOF–WICK* dressing; Johnson & Johnson Medical Ltd., Code 2375), a standard treatment for chronic wounds in the USA. Samples of both test materials were cut using 6 mm-punch biopsies, and weighed to ensure equivalence. To eliminate differences in protease activities due to absorption, the materials were pre-wetted with PBS (0.5 ml; phosphate buffered saline) prior to the addition of the wound fluid; 100 μl was added to each sample of material. The combined mixture was incubated at 37 °C. A 5 ml aliquot was removed from each test sample in triplicate, at the beginning of this incubation period ($t = 0$) and at timed intervals over the next 24 h ($t = 1$, 2, 4 and 24 h). These aliquots were tested immediately for residual enzymic activity.

Collagenase activity was assessed by measuring the ability of this protease to degrade collagen using a fluorimetrically labelled peptide containing a similar cleavage site. Gelatinases present in the wound fluid can then further degrade the newly formed peptides to amino acids and release the fluorigenic label. The substrate, succinyl–glycine–proline–leucine–glycine–proline 7-amino 4-methyl coumarin (Bachem Ltd., UK), when cleaved released the fluorogenic reporter group, 7-amino 4-methyl coumarin. The rate of production of this fluorescent compound was monitored using a microtitre plate fluorimeter (emission 455 nm, excitation 380 nm) and directly relates to the collagenase activity present in the sample. Activity was expressed either as relative fluorescence units per minute (RFU/min) or change in fluorescence when corrected for total protein (RFU/min/mg protein). Each sample was tested six times and the average value calculated. The substrate was prepared at a 10 mM stock concentration, and diluted to a working concentration of 0.5 mM in the assay buffer. The reaction

mixture, combined in a microtitre well (black, flat bottomed) comprised 5 μl wound fluid, 175 μl assay buffer (40 mM Tris/HCl, pH 7.4 containing 200 mM NaCl and 10 mM $CaCl_2$) and 20 μl substrate (final concentration 50 μM). The microtitre plate was read immediately at 455 nm (excitation 383 nm) and at timed intervals over the next hour; between readings the plate was covered and incubated at 37 °C.

2.4. Binding of gelatinases to test samples

A punch biopsy (6 mm) of ORC/collagen was incubated with 200 μl of diluted wound fluid at 37 °C for 2 h, after which a 10 μl aliquot was removed and analysed by gelatin zymography for residual gelatinase activity (both MMP-2 & MMP-9).

Gelatin zymography is a sensitive technique, which allows the visualisation of both active, and proenzyme forms of the gelatinases [27]. Samples for analysis were prepared by diluting in non-reducing Laemmli sample buffer, followed by electrophoresis through a 10% polyacrylamide minigel containing gelatin (0.25 mg/ml), run under non-reducing conditions. Once the sample was electrophoresed and the individual proteins resolved, the gel was washed in 2.5% Triton X-100 for 1 h to remove the SDS. This wash solution was then replaced with assay buffer, 0.04 M Tris/HCl, pH 7.5 containing 0.2 M NaCl and 0.01 M CaCl, and the gel incubated overnight at 37 °C for optimum gelatinase activity. The gel was then stained with Coomassie Brilliant Blue R250 (0.2%, dissolved in 50% methanol/10% acetic acid) for 2 h, and destained in 20% methanol/10% acetic acid. The staining and destaining steps were carried out at room temperature and with gentle shaking to allow even uptake of the protein dye. Areas of substrate digestion were visualised as non-stained regions of the gel and represented the particular enzymatic species and its relative activity. Densitometry was carried out using Sigma Scan software and this was used to compare the levels of gelatinase activity, however, it should be noted that this is only a semi-quantitative measurement.

2.5. Absorption of free radicals by test materials

The ability of the materials to react with and remove oxygen containing free radicals was assessed by the diphenylpicryl hydroxyl (DPPH) test adapted from that described by Blois [28] and Banda et al. [29]. Briefly, 40 mg of the test material was suspended in 2.5 ml of a phosphate buffer (0.1 M; pH 7.0) containing DPPH (10^{-4} M; stock prepared in methanol) and the mixture was shaken and stored in the dark at room temperature. The change in colour was monitored spectrophotometrically at 524 nm over a 6 h period. The effect of different concentrations of test material on free radical absorption was also examined by changing the weight to volume ratio of the incubation mixture (0.05, 0.1, 0.2, 0.4 and 0.8%, w/v). Materials tested in this assay system were ORC/collagen, collagen/alginate and a freeze-dried carboxymethyl–cellulose sponge, prepared by freeze-drying a CMC dispersion in water (1%, w/v).

2.6. Iron and zinc binding studies

The ability of materials to sequester iron and/or zinc ions in an aqueous media was determined as follows. Test samples (100 mg) were incubated in a Tris/HCl buffer (0.1 M, pH 7.4 solution containing 10% calf serum, $FeCl_3 \cdot 6H_2O$ and $ZnSO_4 \cdot 7H_2O$, each at 50 ppm) at 37 °C for 16 h with gentle agitation. The samples were then centrifuged and an aliquot of the supernatant (0.9 ml) was removed and analysed. Protein was removed from the sample of supernatant by trichloroacetic acid (TCA) precipitation: 0.1 ml of 20% TCA (w/v) to give a final concentration of 2% TCA (w/v). After mixing the solution was centrifuged for 15 min and 0.5 ml of supernatant was transferred to a clean dry HPLC vial. To this 0.5 ml of 0.5 M nitric acid was added and samples were analysed for the presence of free iron and zinc in a Dionex DX500 HPLC apparatus. HPLC conditions were as follows—column: Ionpac CS5A Analytical Column (Dionex part no. 046100); Guard Column: Ionpac CS5A (Dionex part no. 046104); eluents: A deionised water, B Metpac PDCA reagent concentrate (part no. 046088), C Metpac PAR reagent diluent (Dionex part no. 046094); postcolumn reagent: 4-(2-pyridylazo) resorcinol monosodium salt (PAR) 0.12 g/l; detector wavelength: 530 nm; temperature: 25 °C; flow rate: 1.2 ml/min (80% eluent A: 20% eluent B) in column; 0.6 ml/min eluent C postcolumn; sample: 100 μl.

Test materials include ORC/collagen, collagen (1%, w/v), ORC and collagen/alginate.

2.7. Platelet-derived growth factor binding studies

The ability of test materials to bind and release PDGF in a physiological environment was accessed by incubating these samples with platelet-derived growth factor-AB (PDGF-AB; R&D Systems Europe, Oxon, UK). Test samples include collagen (0.55%, w/v); ORC/collagen and ORC (INTERCEED*; ETHICON Inc., New Jersey, USA). Due to the acidic nature of ORC, it was necessary to first neutralise this material in order to eliminate pH as a reason for any observed differences (1 ml 0.1 M sodium phosphate dibasic solution for 10 mg ORC material, incubated for 30 min. at room temperature). All test samples (10 mg) were washed with physiological buffered saline (PBS) and incubated in the presence of 2% (w/v) BSA in PBS (1 ml per sample for 1 h at room temperature) prior to initiating binding study. This reduces non-specific binding to the sample and to the surface of the vial and simulates the serum proteins present in wound fluid. Growth factor was then added to each sample—1 ml of PDGF-AB solution (100 ng/ml 0.2% (w/v) BSA/PBS) and incubated for 2 h at 37 °C. An aliquot of this solution pre- and post-incubation was retained for analysis in order to accurately determine levels of PDGF-AB present. The growth factor solution was carefully removed from each test material and stored at 4 °C until analysed. Test materials were then subjected to a PBS wash followed by a series of washes of increasing salt concentrations (0.3, 0.5, 1.0, 2.0, 3.0 and 4.0 M NaCl in PBS and containing 0.2% (w/v) BSA). Bovine serum albumin was included in all solutions to stabilise any growth factor present. Each wash was carried out twice for 15 min at room temperature, after which it was removed and retained for PDGF-AB quantification. The washes obtained from each test material ($n = 6$) were pooled to give two samples that were analysed at two dilutions (5- and 10-fold) in duplicate. PDGF-AB was quantified using an ELISA kit supplied by R&D Systems Europe (Quantikine^{TM} Human PDGF-AB Immunoassay). This kit is supplied complete with all required solutions and instructions for use were followed. The level of PDGF-AB present in each solution was calculated from a standard curve ranging from 0 to 2000 pg/ml.

The Wilcoxon rank sum test was used as a non-parametric test to compare the ability of collagen/ORC to bind PDGF-AB versus either of its individual components. In this test, the data was ranked in order, from the smallest to the largest and the sum of the ranks for each material calculated. To obtain statistical significance, $P < 0.05$, with $n = 4$, the smallest rank sum should be 10 or less.

2.8. Growth factor protection from enzymatic degradation

The ability of ORC/collagen to protect growth factors from proteolytic degradation was assessed by incubating the test material in the presence of PDGF-BB (RW Johnson Pharmaceutical Research Institute, NJ, USA) and plasmin (Sigma) and measuring residual PDGF-BB activity. Samples of ORC/collagen were cut using 6 mm punch biopsies and wetted with PBS (0.5 ml) prior to the addition of growth factor (PDGF-BB; 100 ng in PBS) and enzyme (plasmin; 0.05 units). Control samples contained PDGF-BB alone and PDGF-BB plus plasmin without ORC/collagen were also prepared. Each test sample was incubated overnight at 37 °C in a final volume of 100 μl and in a buffer for optimal plasmin activity (25 mM Tris/HCl, pH 8.1 containing 0.5% Triton X-100). The growth factor solutions were then removed and the biological activity of PDGF-BB estimated by ELISA (PDGF-BB receptor ELISA; R&D Systems Europe, Oxon, UK). PDGF-BB bound to ORC/collagen was recovered by washing with 1.0 M NaCl/PBS for 1 h, 37 °C, prior to ELISA analysis and total PDGF-BB recovered was estimated by adding bound and free values. This ELISA is supplied with instructions for use, which were followed precisely. Samples were diluted 20-fold and tested in triplicate to ensure an accurate determination of PDGF-BB. The level of PDGF-BB present in each solution was calculated from a standard curve ranging from 0 to 60 ng/ml.

2.9. Protection of PDGF from γ-irradiation by ORC/collagen

PDGF-BB (RW Johnson Pharmaceutical Research Institute, NJ, USA) was incorporated into an ORC/collagen freeze-dried sponge (1% (w/v), 55:45 collagen:ORC) to give a final concentration of PDGF at 10 μg/cm^2. This product was then sterilised by γ-irradiation (25 kGy; ^{60}Co source). The release profile of PDGF-BB from this freeze-dried material

was determined before and after sterilisation by incubating a sample (6 mm punch biopsy) at 37 °C in Dulbecco's Modified Eagles's Medium (1 ml; DMEM) and measuring the biological activity of the releasate. The level of PDGF released from each sample was determined by ELISA (R&D Systems Europe, Oxon, UK). This assay is supplied with instructions for use and PDGF-BB was calculated from a standard curve ranging from 0 to 2000 pg/ml. The biological activity of PDGF-BB released from ORC/collagen/PDGF freeze-dried sponges was estimated by proliferation assays as described below.

A methylene blue proliferation assay was used to quantify cell number and assess the biological activity of PDGF-BB. Briefly, adult human dermal fibroblasts (AHDF) were grown to 95% confluency, trypsinised, counted and seeded in 10% FBS/DMEM at a cell density of 3×10^4 cells/ml in a 96 well microtitre plate (100 μl/well). The cells were allowed to adhere and spread overnight. This conditioned media was removed, the cell monolayer washed with PBS, and the test sample or standard was added ($n = 8$; 100 μl/well). Each sample was diluted depending on the estimated concentration of the PDGF incorporated; this was necessary to enable quantification of PDGF over a linear range. Human recombinant PDGF-BB was used as a standard, diluted in serum free-DMEM and used over a concentration range 0–50 ng/ml. The cells were incubated for a further 3 days at 37 °C, 5% CO_2, after which the media was removed and the cell monolayer fixed with FORMOL saline. The cells were then stained with methylene blue, excess stain removed and the dye eluted with acidified ethanol. The solubilised dye was quantified at 630 nm using a microtitire plate spectrophotometer.

3. Results

3.1. Collagenase-like activity in diabetic wound fluid

Wound fluids obtained from human diabetic foot ulcers were analysed to determine the level of collagenase-like activity. Fig. 1 summarises collagenase-like activity detected in three wound fluid samples as estimated using a fluorimetric substrate assay; activity is expressed as RFU/min. A comparison of the protease levels indicates that the activity varied considerably among patients, with patient 1 containing almost three times the protease activity of patient 3.

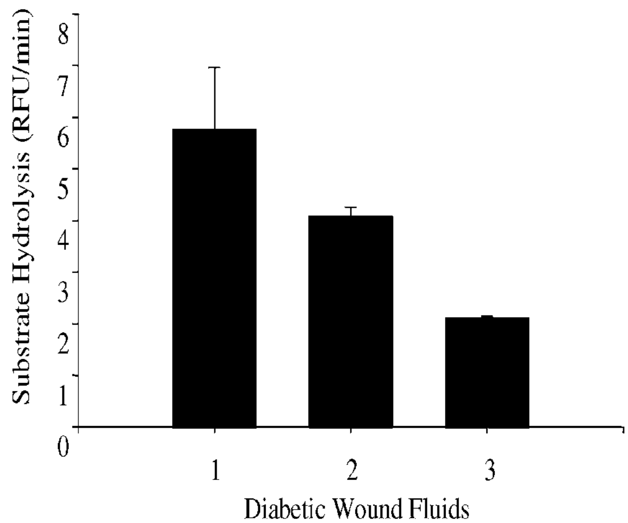

Fig. 1. Levels of collagenase-like activity in the wound fluid collected from foot ulcers in three diabetic patients.

3.2. Effect of ORC/collagen on collagenase-like activity in diabetic wound fluid

The effect of collagen/ORC on collagenase-like activity present in chronic wound fluids was measured by monitoring residual protease activity over time when incubated at 37 °C. Collagenase-like activity was estimated in both the control sample (wound fluid alone) and test samples (wound fluid + ORC/collagen) initially at $t = 0$ and at various timed intervals over the test period of 28 h. In this study, control levels of protease activity decreased over the test period of 28 h, demonstrating the instability of this class of enzyme (Fig. 2). However, when control protease levels were compared with residual levels in the test samples, a marked reduction in activity was observed within the first hour at 37 °C, an effect that was sustained throughout the subsequent 28 h (Fig. 2).

3.3. Binding affinity of ORC/collagen for gelatinises MMP-2 and MMP-9

To examine the ability of collagen/ORC to bind gelatinases, wound fluid samples were analysed at one

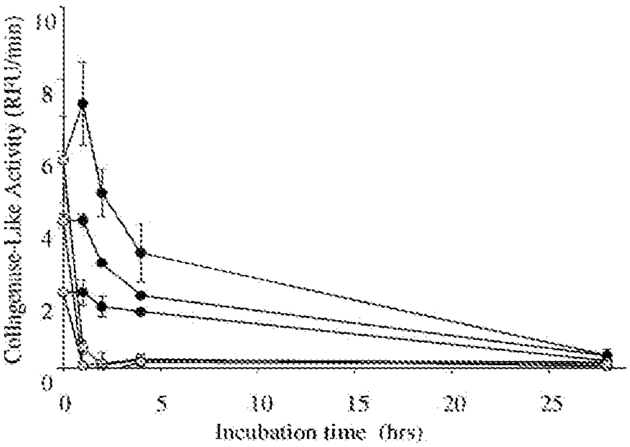

Fig. 2. Effect of ORC/collagen on collagenase-like activity in wound fluids collected from three diabetic foot ulcers. Activity has been estimated using a fluorimetric substrate assay. The graph compares the activity profile of three wound fluid samples in the presence (○) and absence (●) of ORC/collagen.

time point by zymography. A 2 h time point was chosen to avoid deterioration of enzyme activity while allowing time for binding of the proteases. When these wound fluid samples were incubated in the presence of ORC/collagen, a significant reduction in the gelatinases, MMP-2 and MMP-9, was observed in the residual wound fluid (Fig. 3a). Densitometric analysis was used to quantify this reduction in activity and results are represented graphically in Fig. 3b. Higher levels of MMP-9 activity were detected than MMP-2, and the predominant form of both gelatinases was the active form. In each case ORC/collagen significantly reduced gelatinase activity and was found to have a more marked effect on the active forms of the enzyme.

3.4. The binding of free radicals by ORC/collagen

The ability of ORC/collagen to scavenge free radicals was compared with a number of other biopolymers (Fig. 4a). In this assay system, a reduction in absorption at 524 nm is indicative of the reducing potential of the test material, with ascorbic acid constituting the positive control. Of the three biomaterials tested, ORC/collagen was the most effective at reducing DPPH to its hydrazine derivative. Collagen/alginate showed a similar trend but was less effective than ORC/collagen when compared at a similar concentration. No change in absorbance was detected when the DPPH solution was incubated in the presence of a CMC sponge material suggesting that this material would be ineffective at scavenging free radicals.

The reducing potential of ORC/collagen was further investigated in a dose response experiment, where the concentration of ORC/collagen was varied from 0.05 to 0.8% (w/v). The results, shown in Fig. 4b, demonstrate that this biomaterial was as effective as ascorbic acid in reducing DPPH when tested at a optimised concentration.

3.5. Iron and zinc binding studies

The ability of ORC/collagen and its individual components to selectively bind iron and zinc ions in a biological milieu was tested and results are shown in Fig. 5. In this study, residual levels of iron and zinc ions were greatly reduced when incubated in the presence of either ORC (Fig. 5a) or ORC/collagen (Fig. 5b). However, collagen had no obvious effect on these metal ions (Fig. 5c) suggesting that the ability to bind iron and zinc is a characteristic attributed to the polysaccharide component of the ORC/collagen matrix. Collagen/alginate was used as a control material in this experiment and like collagen showed no ability to bind either metal ions (Fig. 5d). This result demonstrates that the binding of iron and zinc ions is a property specific to ORC containing materials.

3.6. Growth factor studies

Growth factors play a pivotal role in wound repair as has been demonstrated by many investigators who have shown that addition of exogenous growth factors can increase tissue synthesis and accelerate the rate of wound repair [6,30]. Thus, in this study, we examined the effect of ORC/collagen, a resorbable matrix, on growth factor binding, release and protection using PDGF as a model growth factor with specific relevance to wound healing.

The ability of ORC/collagen to specifically bind PDGF-AB by physically removing it from solution in the presence of excess protein is shown in Fig. 6a. Collagen and ORC materials alone also show entrapment of PDGF, however, the combination material, ORC/collagen, binds significantly greater amounts of growth factor than its two individual components ($P < 0.05$; Fig. 6a). This growth factor was recovered by

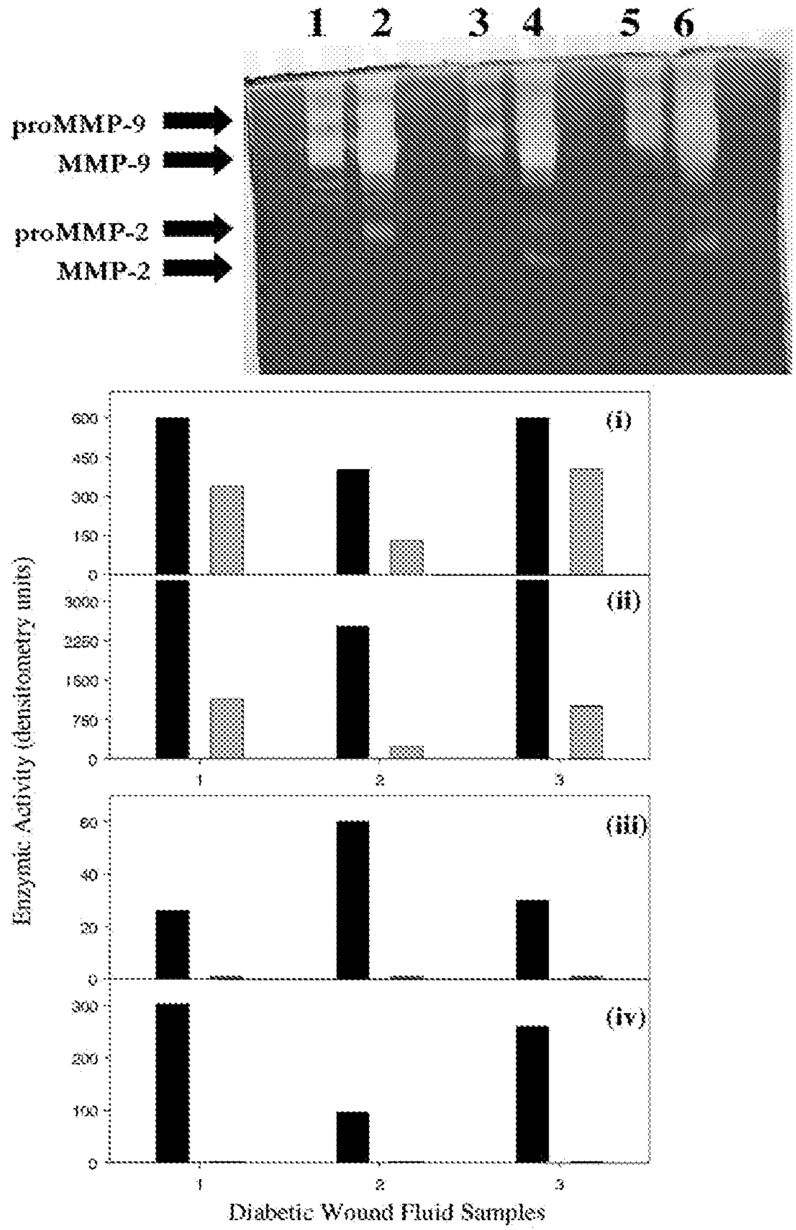

Fig. 3. (A) Gelatin zymogram demonstrating the effect of ORC/collagen on gelatinases levels in wound fluid collected from diabetic foot ulcers. Lanes 2, 4 and 6 represents gelatinase activity in diabetic wound fluid and lanes 1, 3 and 5 show the effect of ORC/collagen on this activity. (B) Relative levels of gelatinase activity is represented graphically as estimated by densiometric analysis: (i) pro MMP-9; (ii) active MMP-9; (iii) pro-MMP-2; (iv) active MMP-2. The effect of ORC/collagen (□) on gelatinase activity was compared with control (■) protease activity levels.

washing the test materials with increasing concentrations of sodium chloride solution suggesting that the interaction is non-covalent and reversible (Fig. 6b). The release profiles for each material is similar with maximal release observed with 0.5 M NaCl washes. The total recovery of PDGF-AB from each material was approximately 70% of original solution; the other 30% was thought to remain bound to the material or to the plastic vial in which the study was performed, since the recovery of PDGF-AB with this ELISA system

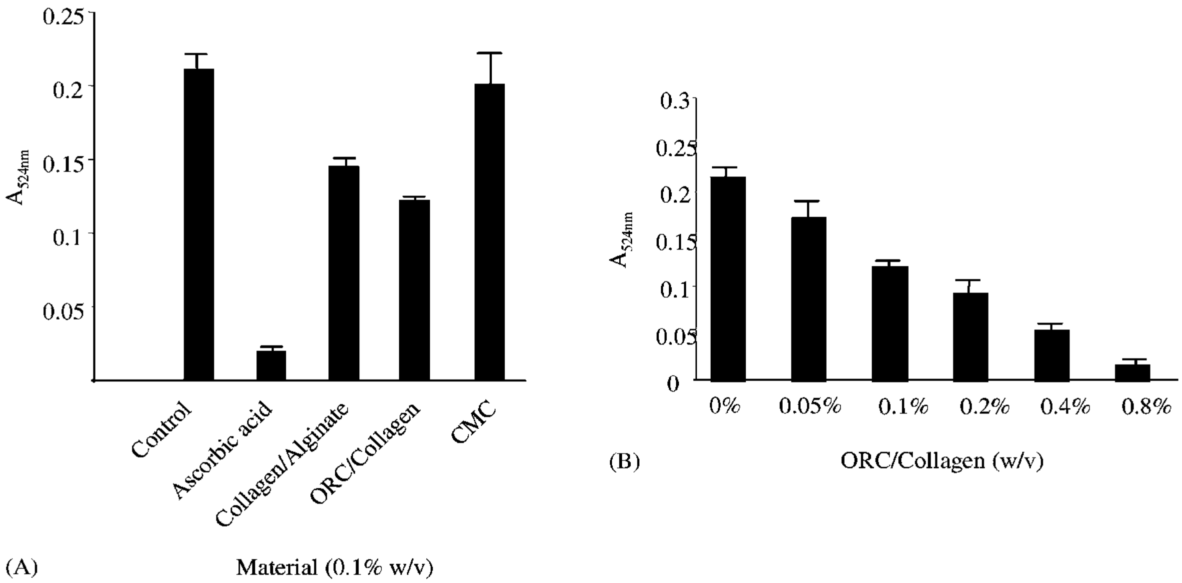

Fig. 4. Effect of biopolymers on scavenging free radicals as estimated by their ability to reduce DPPH to its hydrazine derivative. Graph A shows comparative effect of a number of biopolymers at one concentration; graph B shows a dose response with ORC/collagen.

was estimated to be 98.8% accurate, calculated using a standard solution of growth factor.

The effect of high protease levels present in chronic wound fluid is thought to delay wound repair by degrading newly formed tissue and beneficial proteins such as growth factors. In this study, we have shown that incubating PDGF with plasmin under physiological conditions leads to a reduction in the biological activity of PDGF as assessed by measuring its ability to bind the cell surface receptor (Fig. 7). Incubating plasmin and PDGF in the presence of ORC/collagen preserved this receptor-binding site, and therefore, prevented the loss of biological activity. This suggests that this biomaterial can protect growth factors from enzymatic degradation. PDGF without plasmin was used as a control in this study, however, only 60% activity was recovered demonstrating the instability of this growth factor under physiological conditions.

Additional studies in which PDGF was formulated with ORC/collagen have shown that in this freeze-dried format the growth factor can be successfully sterilised by γ-irradiation. We demonstrated that PDGF was readily released under physiological conditions and retained full biological activity as assessed by cell proliferation (Fig. 8), illustrating that ORC/collagen can be used to protect growth factors from ionising radiation damage and to deliver exogenous growth factors.

4. Discussion

Normal tissue repair is a carefully controlled balance of extracellular matrix synthesis and degradation. However, if proteolysis is excessive or poorly regulated then the balance is shifted towards degradation, which can lead to generalised tissue degradation and non-healing or chronic wounds. In normal wound, repair the activity of these proteases is tightly regulated with control occurring at the cellular level regulating gene expression, and extracellularly regulating enzyme activation and inhibition. A defect in one or more of these control mechanisms would result in an increase in proteolytic activity, a trait of chronic wounds. Whilst it is impossible to determine whether these elevated proteases are responsible for the lack of healing or merely symptomatic of other changes occurring within the wound it has been shown that a decrease in proteolytic activity is concomitant with healing. Thus, it is commonly believed that in this adverse environment, tissue damage and subsequent protease release is a major contributing factor to delayed

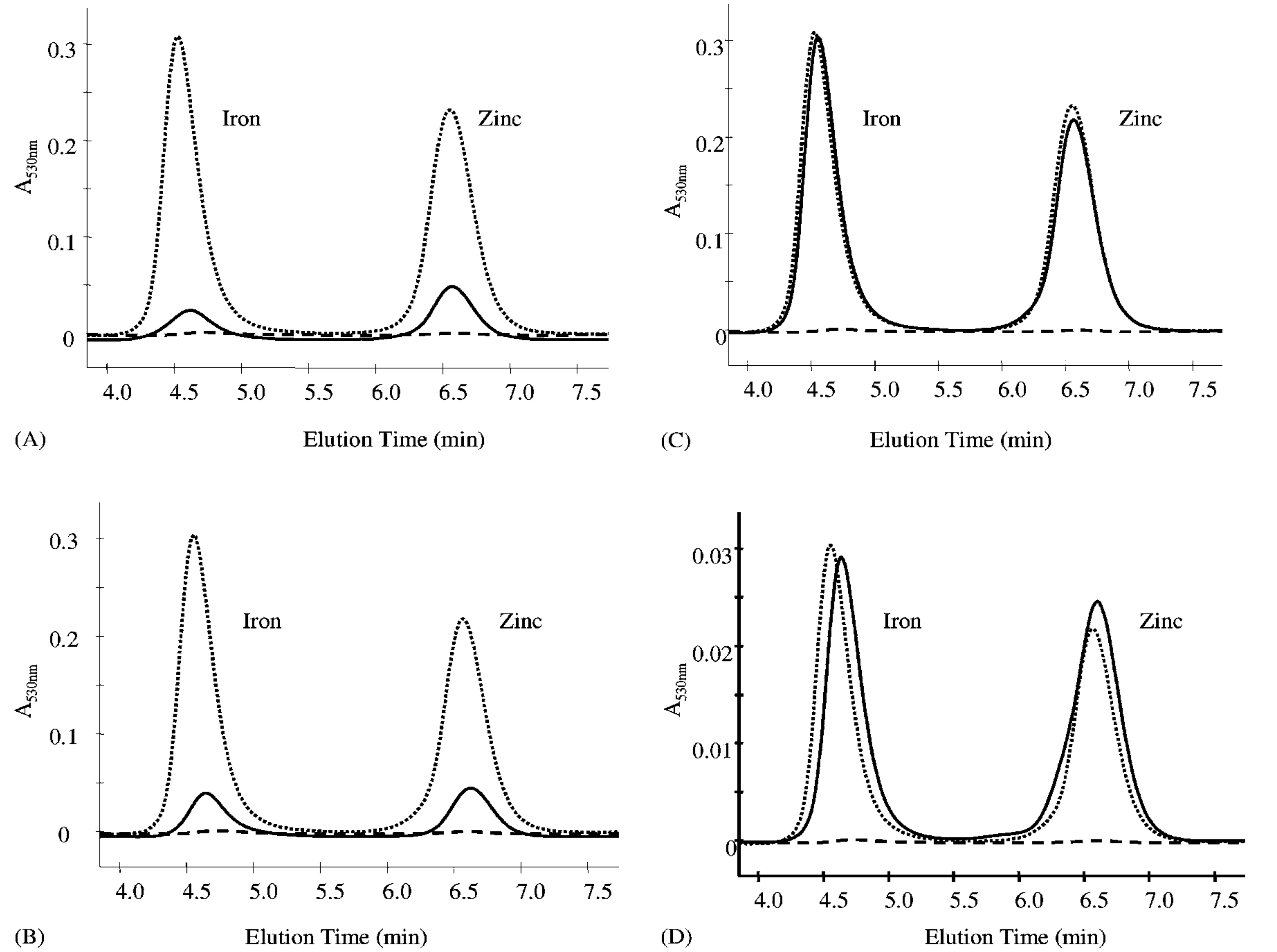

Fig. 5. Elution profiles demonstrating the ability of ORC (A), ORC/collagen (B), collagen (C) and collagen/alginate (D) to selectively bind iron and zinc ions. In all cases, the test material was incubated in a physiological solution containing 10% calf serum and both iron and zinc ions (···); 10% calf serum containing no additional metal ions was used as a negative control (- - -). The ability of the test material to bind the iron and zinc ions is depicted by a solid line (—).

healing rates [31,32]. This hypothesis is supported by several investigators who have examined the biochemical and molecular profiles of human chronic wound tissue and fluids [33,34]. In these studies, they have shown that elevated levels of proteases are present in all types of chronic wounds independent of aetiology. Specifically, these studies have established that chronic wound fluid, in comparison to acute wounds, has abnormally elevated levels of serine proteases; including neutrophil elastase, cathepsin G, urokinase and plasmin [4,35–38] and matrix metalloproteases MMP 1, 2, 3, 8 and 9 [2,3,33,39–43]. It has been postulated that the high protease levels observed in chronic wounds are in part responsible for the paucity of new tissue synthesis through degradation of extracellular matrix components such as fibronectin and vitronectin [32] and indirectly by degrading key regulators of the wound healing process, such as peptide growth factors, e.g. EGF, PDGF, VEGF [7,8,12], and endogenous protease inhibitors TIMP-1 and α-1-antitrypsin [5,33,41]. This may explain why the use of growth factors clinically has met with limited success in the treatment of chronic wounds even when used at high doses [44,45]. We purpose that an alternative strategy for the treatment of chronic wounds would be to reduce the levels of proteolytic enzymes in the wound bed. This would

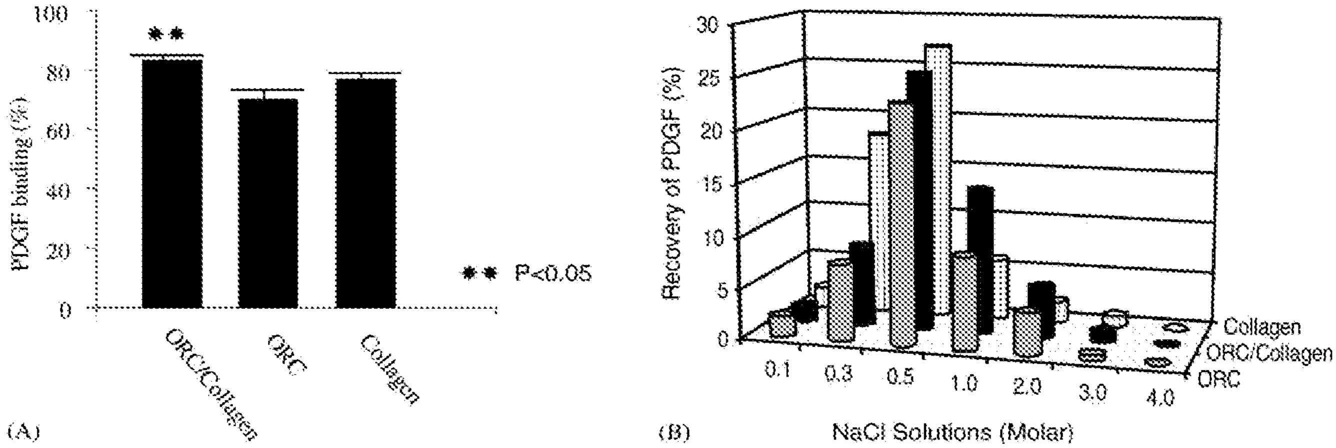

Fig. 6. The ability of ORC/collagen, ORC and collagen to specifically bind PDGF-AB is shown in graph A. The elution profile of this growth factor, from each material, with increasing salt solutions is shown in graph B.

decrease tissue destruction and prevent growth factor degradation, leading to an overall increase in granulation tissue formation and faster wound repair.

In this study, we have shown that ORC/collagen binds and inactivates proteases present in chronic wound fluid obtained from diabetic foot ulcers. The effect was rapid and sustained, a reduction in collagenase-like activity was observed within the first hour and throughout the subsequent 28 h test period. This suggests that ORC/collagen, when applied topically to a chronic wound, could remove excess proteases from the wound bed, and provide a less hostile wound environment thereby reducing tissue destruction and promoting tissue synthesis. The mechanism through which this biomaterial reduces protease activity is unclear, however, the collagen component is likely to act as an alternate or competitive enzyme substrate, while ORC, which is negatively charged under physiological conditions, will bind any positively charged molecules such as metal ions essential for MMP activity.

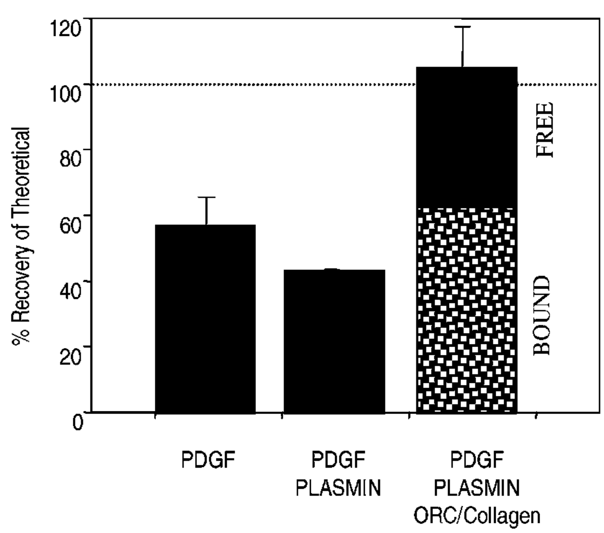

Fig. 7. The ability of ORC/collagen to protect PDGF from enzymatic degradation. PDGF bound to ORC/collagen was estimated after recovery with a 1 M NaCl solution.

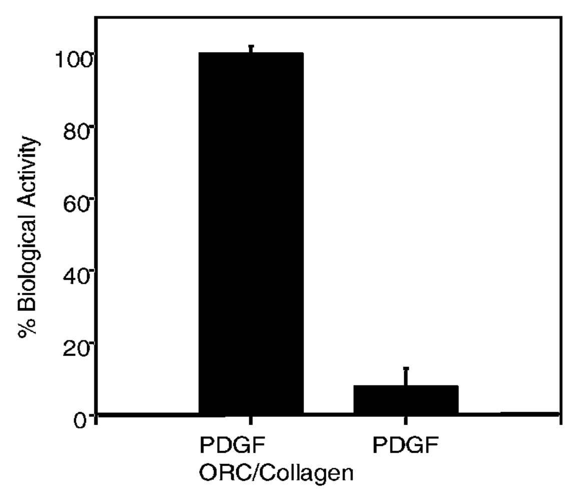

Fig. 8. The ability of ORC/collagen to protect PDGF during γ-irradiation.

In additional studies, we have examined the ability of ORC/collagen to protect PDGF from proteolytic degradation. While this property is in part due to its ability to reduce the level of protease activity, binding studies have shown that ORC/collagen specifically binds PDGF and releases it in a biologically active form as the material degrades. This suggests that ORC/collagen could be used to deliver exogenous, or protect endogenous, growth factors, thereby increasing the amount of biologically active growth factor at the wound site.

Other contributing and potentially inhibitory factors to healing in chronic wounds are free radicals. During acute wound repair they exert a positive effect by attracting macrophages to the wound site and initiating inflammation. However, in chronic wounds an excess of oxygen free radicals are considered detrimental to the healing process, leading to a prolonged inflammatory state and increased proteolysis and tissue degradation [14]. In chronic wounds, the presence of H_2O_2 is converted to ROS, an effect catalysed by the presence of excess iron. These ROS subsequently cause protein and lipid peroxidation and DNA damage, leading to cell disruption, tissue damage and cell death [17,21]. The ability of ORC/collagen to influence the level of free radicals in the chronic wound environment is supported by both direct and indirect evidence. Its ability to directly scavenge free radicals is demonstrated by its ability to reduce DPPH to its hydrazine derivative and by its ability to protect PDGF from free radical damage during γ-irradiation. Demonstrating the ability of ORC/collagen to bind free iron in wound fluid provides indirect evidence to support this hypothesis. Thus, we postulate that ORC/collagen will protect growth factors and extracellular matrix proteins from free radical damage, specifically in environments where they are in excess, such as chronic wounds.

In summary, a chronic wound can be considered as an imbalance between tissue deposition stimulated by growth factors and tissue destruction mediated by proteases. The results presented in this paper demonstrate that ORC/collagen can reduce proteolytic activity, absorb oxygen free radicals, bind excess iron, and protect growth factors present in chronic wound fluid. This suggests that ORC/collagen can redress the imbalance of the chronic wound environment and therefore may have a beneficial effect in the treatment of such wounds.

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